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Report

Differential Effects of Unfolded Protein Response Pathways on Axon Injury-Induced Death of Retinal Ganglion Cells

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SUMMARY

Loss of retinal ganglion cells (RGCs) accounts for visual function deficits after optic nerve injury, but how axonal insults lead to neuronal death remains elusive. By using an optic nerve crush model that results in the death of the majority of RGCs, we demonstrate that axotomy induces differential activation of distinct pathways of the unfolded protein response in axotomized RGCs. Optic nerve injury provokes a sustained CCAAT/enhancer binding homologous protein (CHOP) upregulation, and deletion of CHOP promotes RGC survival. In contrast, IRE/XBP-1 is only transiently activated, and forced XBP-1 activation dramatically protects RGCs from axon injury-induced death. Importantly, such differential activations of CHOP and XBP-1 and their distinct effects on neuronal cell death are also observed in RGCs with other types of axonal insults, such as vincristine treatment and intraocular pressure elevation, suggesting a new protective strategy for neurodegeneration associated with axonal damage.

INTRODUCTION

Retinal ganglion cells (RGCs) are the only neurons that relay visual information from the retina to the brain. These neurons are highly vulnerable when their axons, which collectively form the optic nerve, are damaged (Levin, 1997). For example, traumatic optic nerve injury and subsequent loss of RGCs often occur in the setting of head injury, as a consequence of traffic accidents or falls. In rodents, the majority of RGCs undergo cell death around 2 weeks after intraorbital optic nerve injury (Berkelaar et al., 1994; McKernan and Cotter, 2007; Sellés-Navarro et al., 2001), creating a first hurdle for successful neural repair. In addition to optic nerve trauma, the retinal pathology of different types of optic neuropathy, in particular glaucoma, is also characterized by selective RGC loss (Howell et al., 2007; Kerrigan et al., 1997; Libby et al., 2005; Quigley, 1993; Quigley et al., 1995; Weinreb and Khaw, 2004). In these conditions, RGC loss has been attributed to apoptotic death (Howell et al., 2007; Kerrigan et al., 1997; Libby et al., 2005; Quigley, 1993; Quigley et al., 1995; Weinreb and Khaw, 2004; Qu et al., 2010). However, RGC apoptosis may occur as the last step in these diseases, so that targeting apoptotic effectors may not be an efficient strategy for therapy. Thus, deciphering the key upstream signals that trigger the apoptotic cascade in RGCs should provide important targets for therapeutic interventions.

Multiple stimuli, such as hypoxia, nutrient deprivation, viral infection, and disturbance of calcium levels, can directly or indirectly cause accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER), triggering an ER stress condition which leads to an evolutionarily conserved unfolded protein response (UPR) (Ron and Walter, 2007). The UPR has been proposed to be a protective mechanism that limits ER protein loading by inhibiting protein translation, facilitates protein folding through increasing the expression of ER chaperones, and removes misfolded proteins from the ER through degradation. However, prolonged and unrestrained ER stress could lead to the activation of proapoptotic signaling pathways.

In mammals, the UPR includes three signal-transduction pathways initiated by three ER-resident stress-sensing proteins: protein kinase RNA-like ER kinase (PERK), inositol-requiring protein-1 (IRE1), and activating transcription factor-6 (ATF6). PERK activation leads to the phosphorylation of the eukaryotic inactivation factor 2α (eIF2α). While suppressing general protein translation, eIF2α phosphorylation also promotes the selective translation of some mRNAs, such as ATF4, a transcription factor that induces CCAAT/enhancer binding homologous protein

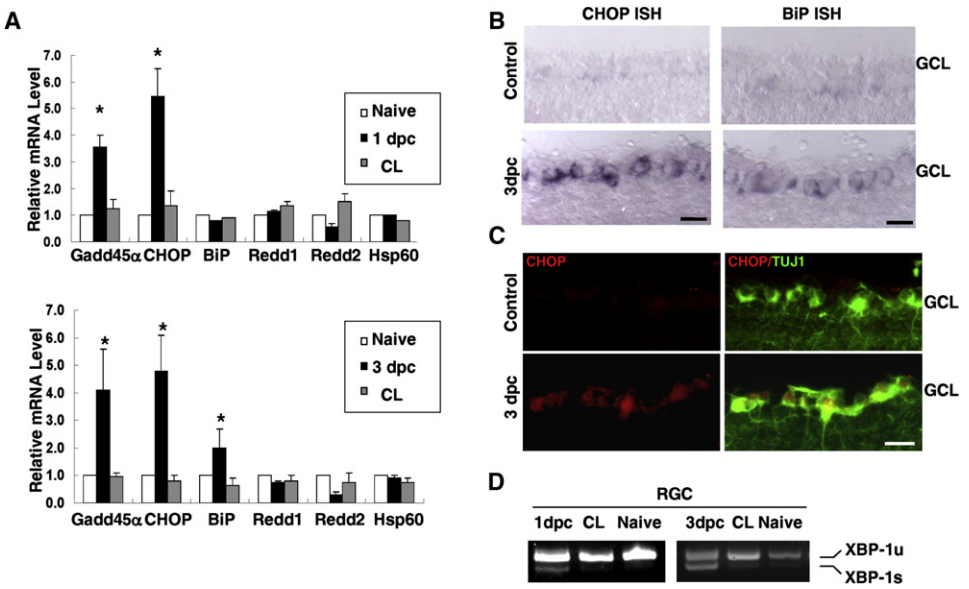

Figure 1. Optic Nerve Injury Induces UPR in Adult RGCs

(A) Quantification of expression of different molecules in RGCs detected by qRT-PCR. Each sample was run in quadruplicate in each assay. GAPDH was used as the endogenous control. CL, contralateral uninjured eye. *$p < 0.01$, paired Student's t test. Data are presented as means ± SEM and n = 6. (B) In situ hybridization (ISH) results showing the expression of CHOP and BiP in the ganglion cell layer of adult mouse retinas. Scale bar represents 20 μm. 3 dpc, 3 days postcrush; GCL, ganglion cell layer. (C) Immunohistochemical analysis for CHOP or TUJ1 immunoreactivity in retinal sections. Scale bar represents 20 μm. (D) Detection of unspliced and spliced XBP-1 mRNA (XBP-1u or XBP-1s) by RT-PCR. The mRNAs were prepared from retrograde-labeled and FACS-purified adult RGCs from retinas 1 day, 3 days postcrush (1 dpc, 3 dpc), contralateral (CL) eyes, and naive eyes. See also Figure S2.

(CHOP) expression, which has been generally used as an ER stress marker (Fels and Koumenis, 2006; Harding et al., 2003). On the other hand, the site-specific endoribonuclease function of IRE1 mediates the specific splicing of XBP-1 mRNA to generate an active (spliced) form XBP-1s (Calfon et al., 2002; Sidrauski and Walter, 1997; Yoshida et al., 2001). XBP-1s targets a set of genes that increases the ER protein-folding capacity and facilitates degradation of misfolded proteins (Lee et al., 2003; Shaffer et al., 2004). Although IRE/XBP-1 has been proposed to be protective, the in vivo effect of XBP-1 on neuroprotection is less clear. In fact, it was shown that XBP-1 deletion in the nervous system (*XBP-1*$^{flox/flox}$ mice crossed with nestin-Cre mice) could extend lifespan of transgenic mice expressing a mutant SOD1, an amyotrophic lateral sclerosis model, by enhancing autophagy, and thus degradation of the mutant SOD1 protein in vivo (Hetz et al., 2009).

In our analysis of the mechanisms for reduced protein synthesis ability in axotomized RGCs in adult mice (Park et al., 2008), we found that axotomized RGCs showed signs of UPR, indicating that ER stress is induced in these neurons. In fact, ER structures that are distributed along entire lengths of axons and are connected with those in the neuronal somas might possess the unique properties of transducing the local axonal signals to the soma of individual neurons. However, despite previous reports about ER stress responses in neurons (Aoki et al., 2002; Saxena et al., 2009), it is unknown how these pathways are activated and, more importantly, what the functional consequences are. Thus, we decided to assess axotomy-triggered UPR in depth using in vivo mouse models.

RESULTS

Axotomy Triggers UPR in RGCs

CHOP, a key downstream target of PERK pathway, has been linked to apoptosis after ER stress in multiple disease models (Pennuto et al., 2008; Puthalakath et al., 2007; Silva et al., 2005; Song et al., 2008; Zinszner et al., 1998). To assess the expression of CHOP in intact and axotomized RGCs, we purified RGCs from wild-type rats with or without optic nerve crush by retrograde labeling and fluorescence-activated cell sorting (FACS) (Park et al., 2008). Through the use of the mRNA isolated from purified RGCs, quantitative real-time PCR (qRT-PCR) analysis showed increased expression of CHOP and other ER stress markers, such as GADD45α (Lee et al., 2003), in axotomized RGCs (Figure 1A), which was further confirmed by both in situ hybridization and immunohistochemical analysis in retinal sections (Figures 1B and 1C). In contrast, Redd1/2, the inhibitors of the mTOR pathway induced by hypoxia (Brugarolas et al., 2004), and Hsp60, a mitochondria stress chaperone (Deocaris et al., 2006), were not induced by axotomy in RGCs (Figure 1A).

We also examined the activation of other UPR targets in axotomized RGCs. Because XBP-1 splicing has been considered as a hallmark of UPR (Calfon et al., 2002; Sidrauski and Walter, 1997; Yoshida et al., 2001), we examined whether the splicing product of XBP-1 (XBP-1s) could be detected in RGCs after optic nerve injury. By RT-PCR with mRNAs from purified RGCs, we found that a small amount of XBP-1s appeared in the RGCs obtained at both 1 and 3 days after optic nerve crush,

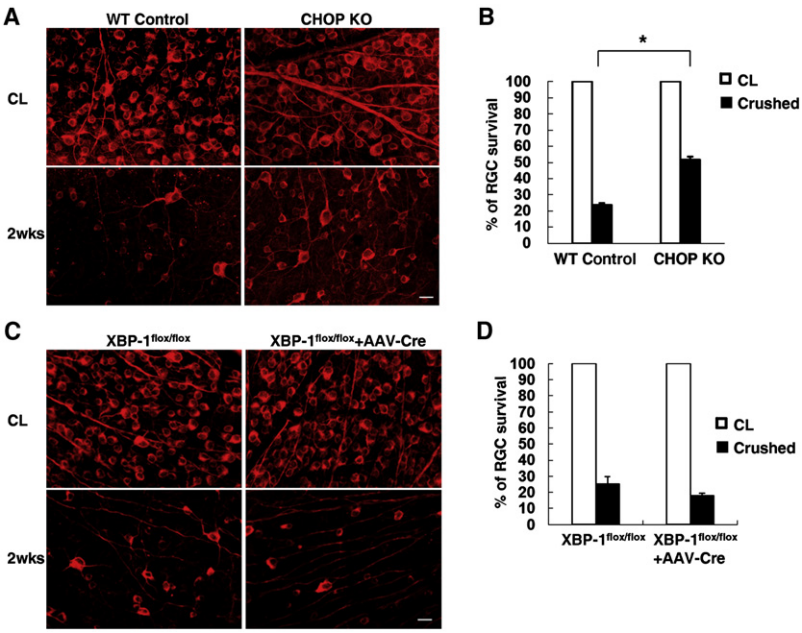

Figure 2. *CHOP* KO, but Not *XBP-1* KO, Increases RGC Survival after Optic Nerve Injury

(A and C) Representative images of fluorescent photomicrographs of flat-mounted retinas showing surviving TUJ1$^+$ RGCs at 2 weeks after injury in WT mice and CHOP KO mice (A) and *XBP-1*$^{flox/flox}$ control mice (no AAV-Cre) and *XBP-1*$^{flox/flox}$ mice injected with AAV-Cre (C). CL, contralateral uninjured eye. Scale bar represents 20 μm. (B and D) Quantification of surviving RGCs, represented as percentage of TUJ1$^+$ RGCs, compared to the uninjured contralateral retinas, in *CHOP* KO mice (B) or *XBP-1* KO mice (D). *$p < 0.01$, Student's t test. Data are presented as means ± SEM and n = 6. See also Figure S1.

but not in those of naive mice (Figure 1D). Consistently, modest upregulation of BiP, a XBP-1 target (Lee et al., 2003), was seen at 3 days postinjury (Figures 1A and 1B), consistent with a modest activation of the IRE1/XBP-1 pathway in axotomized RGCs. These results suggested that optic nerve injury triggers robust CHOP induction and modest XBP-1 activation in axotomized RGCs.

CHOP Deficiency Promotes RGC Survival

We next examined whether UPR activation contributes to RGC cell death after axotomy. We thus performed optic nerve crush in *CHOP* knockout (KO) mice (Marciniak et al., 2004) and control mice and analyzed the extents of RGC survival by counting survived TUJ1-positive RGCs at different postinjury points (Park et al., 2008). Consistent with the notion that CHOP could act as a proapoptotic molecule, we found significant increases of RGC survival in *CHOP* KO mice, compared to wild-type (WT) control mice, after injury (Figure 2A). As shown in Figure 2B, 52% of RGC survived in *CHOP* KO mice 2 weeks after optic nerve crush, compared to 24% RGC survival in WT mice. Therefore, these results suggest that CHOP activation is a critical mechanism that mediates axotomy-induced RGC death.

Lack of Significant Effects of XBP-1 Deletion on RGC Survival

Based on the observation of XBP-1 activation, albeit to a modest level, in axotomized RGCs (Figure 1D), we examined the effects of genetic deletion of XBP-1 in RGCs on RGC survival after optic nerve injury. Because *XBP-1* germline KO is embryonic lethal (Reimold et al., 2000), we utilized an adeno-associated virus (AAV)-Cre-assisted conditional knockout strategy (Park et al., 2008) to delete *XBP-1* in adult RGCs of *XBP-1*$^{flox/flox}$ mice (Hetz et al., 2008). Intravitreal injection of AAV-Cre has previously been shown to delete a floxed gene in most RGC (Park et al., 2008). By in situ hybridization, we further verified the lack of XBP-1 expression in the RGCs of *XBP-1*$^{flox/flox}$ mice with AAV-Cre injection (see Figure S1A available online). As shown in Figures 2C and 2D, there was no significant difference in RGC survival between *XBP-1*-deleted mice and control mice after injury, suggesting that XBP-1 deletion does not affect axotomy-triggered RGC death.

Differential Activation of CHOP and XBP-1 in RGCs after Axonal Insults

To explore possible mechanisms for differential effects of CHOP and XBP-1 deletion on RGC death, we monitored the temporal expression levels of XBP-1s and CHOP in axotomized RGCs during the first week after axotomy (because of difficulty in collecting RGCs at later time points due to massive RGC loss). XBP-1s level was elevated in RGCs isolated from animals at 3 and 5 days after optic nerve crush, but reduced at 7 days postinjury (Figure S1B). In addition to being transient, XBP-1 activation in axotomized RGCs is also modest, when compared with the amount of XBP-1s in cells treated with thapsigargin, an agent that induces ER stress by depletion of luminal calcium stores (Figure S1B). In contrast, CHOP expression was persistently high during the time course studied (Figure S1C). These results suggest that optic nerve injury triggers differential activation of different UPR pathways: although CHOP is robustly and persistently activated, XBP-1 is only transiently and modestly activated. Because previous studies in cultured nonneuronal cells suggested that the duration of IRE1/XBP-1 activation correlates with its protective effects (Lin et al., 2007), the transient activation of IRE1/XBP-1 in axotomized RGCs might explain the failure of XBP-1 knockout in affecting RGC survival.

To assess whether differential activation of CHOP and XBP-1 occurs in other types of axonal damage, we intravitreously injected vincristine, a microtubule destabilizer which preferentially induces axonal degeneration (Silva et al., 2006; Vohra et al., 2010). As shown in Figure S2, vincristine triggered CHOP upregulation, but not XBP-1 splicing (detected by RT-PCR using mRNAs of whole retina or isolated RGCs), at 1 day postinjection. In contrast, both CHOP upregulation (Figure S2A) and XBP-1

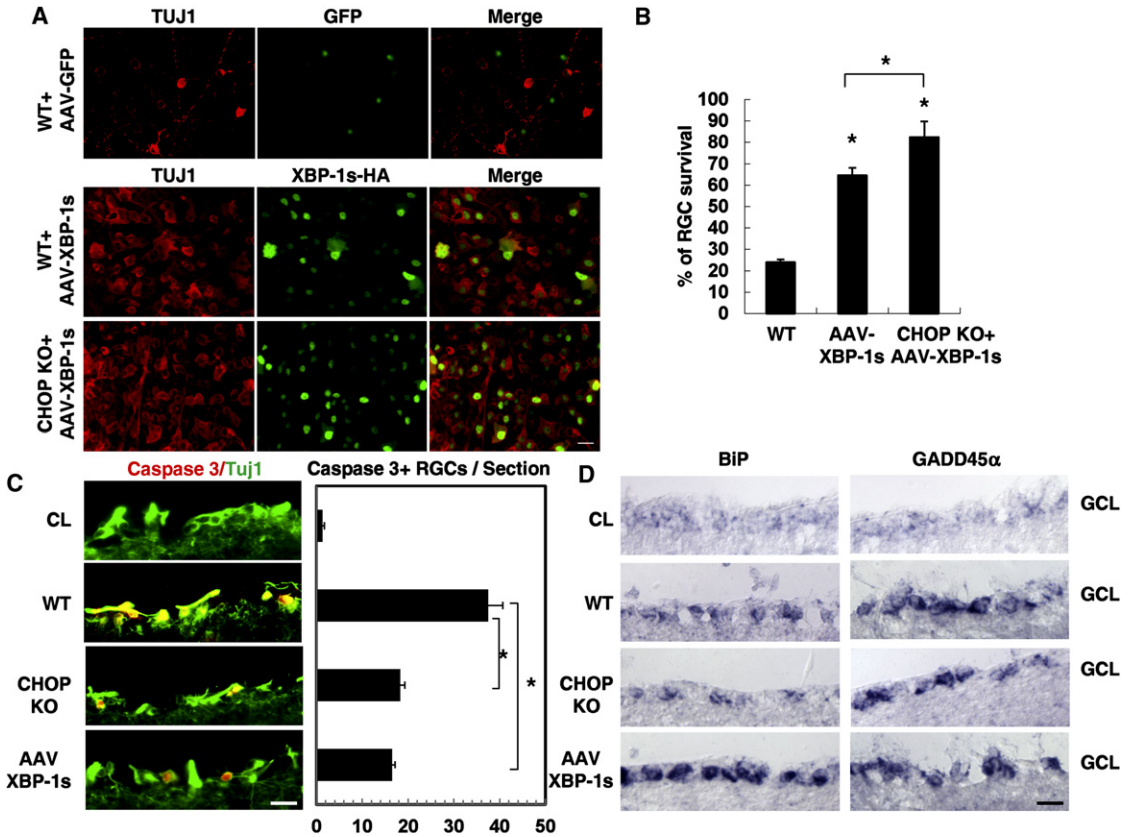

Figure 3. The Effects of XBP-1s Overexpression on RGC Survival after Optic Nerve Injury

(A) Representative images of flat-mounted retinas showing surviving TUJ1$^+$ RGCs at 2 weeks postinjury in WT control mice injected with AAV-GFP or AAV-XBP-1s or *CHOP* KO mice injected with AAV-XBP-1s. Anti-HA antibody is used to indicate RGCs injected with AAVs expressing HA-tagged XBP-1s. Scale bar represents 20 μm. (B) Quantification of TUJ1$^+$ RGCs represented as percentage of TUJ1$^+$ RGCs compared to the uninjured contralateral retinas. Data are presented as means ± SEM and n = 6. *p < 0.01, one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. (C) Left: immunostaining of active caspase-3 at 3 days postinjury in WT mice, CHOP KO mice, and AAV-XBP-1s injected mice. CL, contralateral uninjured eye. Scale bar represents 20 μm. Right: quantification of caspase-3$^+$ RGCs per section. Data are presented as means ± SEM and n = 4. *p < 0.01, one-way ANOVA and Tukey's multiple comparison test. (D) In situ hybridization results showing the expression of BiP and GADD45α in the ganglion cell layer (GCL) of WT mice, *CHOP* KO mice, and AAV-XBP-1s injected mouse retinas 3 days postinjury. CL: contralateral uninjured eye. Scale bar represents 20 μm. See also Figure S3.

splicing (Figure S2B, detected by RT-PCR using mRNAs from whole retinas) were induced by similarly applied thapsigargin, which presumably acts on both axons and cell bodies. Thus, instead of simultaneous activation of all UPR pathways that occur in nonneuronal cells, axonal insults preferentially lead to the activation of CHOP, but not XBP-1, in RGCs. In comparison with other cell types, a striking feature of neurons is the unique compartmentation in which the axon is separated from the soma. It is conceivable that certain unique properties of the axonal compartment, such as the lack of detectable mRNAs and the long distance to the soma, might contribute to the limited XBP-1 activation in axotomized adult RGCs.

Overexpressing XBP-1s Promotes RGC Survival after Optic Nerve Injury

Limited XBP-1 activation in axotomized RGCs suggests the possibility that forced XBP-1 activation might alter RGC survival after optic nerve injury. To test this, we overexpressed an active hemagglutinin (HA)-tagged XBP-1s in RGCs using recombinant AAVs in WT and *CHOP* KO mice. As shown in Figure S3A, approximately 50% and 80% of TUJ1-positive RGCs were stained with an anti-HA antibody 1 or 2 weeks after injection of AAV-XBP-1s-HA, respectively. Then, we performed optic nerve injury at 2 weeks after the intravitreal injection of AAVs. As shown in Figures 3A and 3B, AAV-XBP-1s dramatically increased RGC survival in both WT mice and *CHOP* KO mice. In comparison with approximately 20% RGC survival in AAV-green fluorescent protein (GFP) injected control mice, WT mice with XBP-1s overexpression showed approximately 64% RGCs survival at 2 weeks after injury (Figure 3B). *CHOP* KO could further enhance the survival effects of XBP-1s overexpression, because 82% RGCs survived in *CHOP* KO mice with XBP-1s overexpression 2 weeks after optic nerve crush (Figure 3B). Taken together, these results suggest that XBP-1 and CHOP play opposite roles in controlling neuronal survival after axonal injury.

Because failure of RGC axon regeneration is another major feature of optic nerve damage, we also determined whether increase of RGC survival improves axon regeneration. We

anterogradely labeled the RGC axons with neuronal tracer cholera toxin B; however, in all of these animals, we failed to observe any enhancement of optic nerve regeneration (Figure S3B), suggesting that UPR selectively affects neuronal survival, but not axon regeneration.

Independent Action of XBP-1 and CHOP on RGC Survival

We next examined possible interactions between XBP-1 and CHOP in their effects on neuronal survival. Although the promoter of *CHOP* contains a putative XBP-1 binding site (Roy and Lee, 1999; Urano et al., 2000), we failed to observe significant change of CHOP expression in intact or injured RGCs upon AAV-assisted XBP-1s overexpression (Figures S3C and S3D). Conversely, XBP-1s induction was not affected by *CHOP* knockout (Figure S3E), suggesting independent regulation of XBP-1 and CHOP activation or expression in neurons.

Both *CHOP* KO and XBP-1s overexpression reduced the extent of injury-induced RGC apoptosis, as indicated by TUNEL (data not shown) and active caspase-3 staining (Figure 3C). We then assessed whether similar down-stream effectors might contribute to the effects of *CHOP* KO and XBP-1s overexpression on neuronal survival. As shown in Figure 3D, neither *CHOP* KO nor XBP-1s overexpression altered axotomy-induced expression of GADD45α. However, XBP-1s overexpression, but not *CHOP* KO, significantly induced the expression of the ER chaperon BiP (Lee et al., 2003), suggesting that different downstream mechanisms might be involved in the effects of XBP-1s and *CHOP* KO on regulating RGC apoptosis after axon injury.

The Effect of CHOP and XBP-1 on Glaucomatous RGC Degeneration

Glaucoma is a common form of optic neuropathy that is characterized by progressive RGC degeneration (Howell et al., 2007; Kerrigan et al., 1997; Libby et al., 2005; Quigley, 1993; Quigley et al., 1995; Weinreb and Khaw, 2004). Elevated intraocular pressure (IOP) is the most recognized risk factor for primary open-angle glaucoma (Quigley, 1993). Studies in primates demonstrate that experimentally elevated IOP results in axonal transport obstruction and nerve damage at the optic nerve head, followed by RGC loss (Minckler et al., 1977). Moreover, it was shown that elevated IOP induces CHOP expression in RGCs (Doh et al., 2010). We thus attempted to examine whether manipulation of the UPR pathways could protect RGCs in a mouse model of glaucoma in which IOP was elevated by injection of microbeads into the anterior chamber of adult mice to block aqueous outflow (the contralateral eyes with sham injection served as controls) (Sappington et al., 2010). This established procedure has been shown to induce many features of glaucoma, such as optic nerve head cupping, optic nerve degeneration, and RGC loss (Chen et al., 2010; Sappington et al., 2010).

A first anterior chamber injection of microbeads induced IOP elevation to 20–25 mmHg, about two times higher than normal, for 4 weeks, and then elevation was maintained for another 4 weeks with a second microbead injection, compared to a steady level of 10 mmHg of IOP in the control eyes (Figure 4A). As a result, approximately 42% RGCs are lost at 8 weeks after microbead injection in WT mice (Figures 4B and 4C). In these animals, at 7 days after microbead injection, there was a marked increase in CHOP expression in RGCs assessed by immunostaining (Figure S4A). However, we failed to detect the spliced form of XBP-1 at all the time points studied (3, 5, and 7 days after microbead injection) (data not shown), suggesting that similar to optic nerve injury, IOP elevation triggers differential activation of different UPR pathways in RGCs.

Importantly, both *CHOP* KO and XBP-1s overexpression significantly reduced RGC death. The combination of *CHOP* KO and XBP-1s overexpression showed a trend of further protection, but the extent of the protection did not reach the level of statistical significance as compared to *CHOP* KO or XBP-1s overexpression alone (Figures 4B and 4C). These protective effects are not due to the alteration of the IOP levels, because microbead injection induced similar degrees of IOP elevation in all experimental groups (Figure S4B). Because brain-derived neurotrophic factor (BDNF) has been shown to be protective for RGCs (Cohen-Cory and Fraser, 1994; Mansour-Robaey et al., 1994), we simultaneously applied BDNF and XBP-1s to the eyes of animals that received an optic nerve crush injury (Figure S4C) or were subjected to IOP elevation (Figure S4D). Although BDNF alone protected RGCs to some extent, it did not lead to a significant further enhancement of RGC survival in any of these models when it was combined with XBP-1s overexpression. The mechanistic interactions between UPR and neurotrophin pathways remain to be further elucidated.

To mimic a clinically relevant scenario, we also examined whether a delayed expression of XBP-1s can be protective for RGCs in the IOP-elevated model. We thus increased IOP by microbead injection followed by introduction of AAV-XBP-1s 1 or 7 days later. Because AAV-mediated gene expression in RGCs is normally peaked at 2 weeks after infection (Martin et al., 2002; Park et al., 2008), XBP-1s expression in RGCs is likely to occur 2–3 weeks after IOP elevation. Interestingly, such delayed AAV-XBP-1s expression still showed significant protective effects on RGCs (Figures 4D and 4E), suggesting that forced XBP-1s expression might be a promising therapeutic approach for RGC degeneration in glaucoma.

DISCUSSION

A predominant hypothesis holds that ER stress activates all UPR pathways, thereby simultaneously producing antagonistic outputs that can be both protective and harmful to cells; only unresolved ER stress results in cell death (Ron and Walter, 2007). Although these principles might be applicable to non-neuronal cells, the results from this study suggest a different scenario for neurons, at least adult RGCs. By using different axonal damage models, we demonstrate that diverse UPR pathways are differentially activated in the affected RGCs and in fact have opposite effects on neuronal survival. These results reveal a potentially important logic of protecting RGCs by differentially manipulating the UPR pathways.

In all models, we observed robust and persistent CHOP induction. Consistent with previous studies (Pennuto et al., 2008; Puthalakath et al., 2007; Silva et al., 2005; Song et al., 2008; Zinszner et al., 1998), CHOP induction might be an important

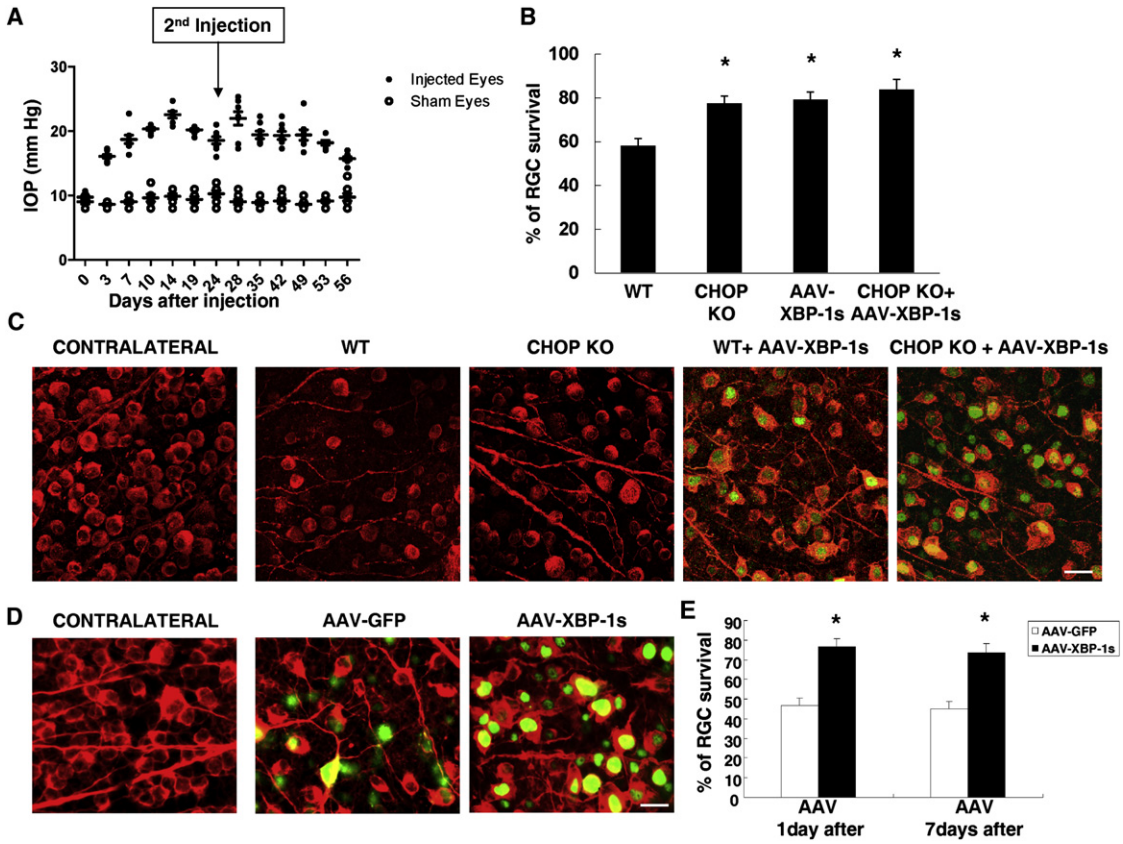

Figure 4. CHOP Deletion and XBP-1s Overexpression Protect RGCs after IOP Elevation

(A) Measured IOP levels in the eyes of the wild-type mice with saline injection (sham eyes) or microbead injection (injected eyes). Whereas the sham eyes exhibited a steady level of IOP at 10.0 ± 1.5 mmHg (n = 8), a single injection induced IOP elevation that lasts for 4 weeks, and a second dose of microbead injection on day 24 (arrow) could maintain elevated IOP levels for 8 weeks (n = 8). (B) Quantification of surviving RGCs, represented as percentage of TUJ1$^+$ RGCs, compared to the uninjured contralateral retinas, in each group at 8 weeks after IOP elevation. Data are presented as means ± SEM and n = 8. *p < 0.01; one-way ANOVA and Tukey's multiple comparison test. (C) Representative images of TUJ1 (red) and HA (green) labeling in flat-mounted retinas from uninjected contralateral eyes and from those eyes 8 weeks after IOP elevation. AAV-XBP-1s was injected 2 weeks before IOP elevation. Scale bar represents 20 μm. (D) Representative images of TUJ1 (red) and GFP or HA (green) labeling in flat-mounted retinas from uninjected contralateral eyes and from those eyes 8 weeks after IOP elevation. AAV-XBP-1s was injected 1 day after IOP elevation. Scale bar represents 20 μm. (E) Quantification of surviving RGCs, represented as percentage of TUJ1$^+$ RGCs, compared to the uninjured contralateral retinas, in each group at 8 weeks after IOP elevation. AAV-XBP-1s was injected 1 day or 7 days after IOP elevation. Data are presented as means ± SEM and n = 8. *p < 0.01; Student's t test. See also Figure S4.

contributor to RGC loss in these conditions. In contrast, in these same models, IRE/XBP-1 pathway either is not activated or is only transiently activated, consistent with the lack of phenotypes of XBP-1 deletion on neuronal death. Directly overexpressing an active XBP-1 in the adult RGCs protects RGCs from apoptotic death after both acute and chronic insults, indicating a neuroprotective role of XBP-1 in RGC survival.

Probably, all of the ER stress sensors, including IRE1, become activated when axon injury occurs. The unique properties of the axonal compartments, such as length and limited mRNAs localization, might explain the different UPR activation patterns in adult RGCs (this study) and nonneuronal cells (Ron and Walter, 2007). For example, because activation of XBP-1, a protective arm of UPR pathways, requires IRE1-mediated mRNA splicing (Yoshida et al., 2001), little XBP-1 mRNAs in the axonal compartment in adult neurons might limit the activation of this pathway in the axon. As a consequence, axonal insults result in the overweight of proapoptotic UPR activation, which might contribute to irreversible neuronal death associated with traumatic optic nerve injury, glaucoma, and perhaps other types of neuropathies. In light of recent successes in AAV-mediated gene therapy in retinal diseases (Busskamp et al., 2010; Tan et al., 2009), our results may provide potentially important molecular targets for neuroprotective strategies for optic nerve injury and diseases.

EXPERIMENTAL PROCEDURES

Detailed methods and materials are in the Supplemental Experimental Procedures.

Mice

CHOP KO and C57BL/6 mice and Sprague-Dawley rats were purchased from the Jackson Laboratory. *XBP-1$^{flox/flox}$* mice were described as before (Hetz et al., 2008). All experimental procedures were performed in compliance

with animal protocols approved by the Institutional Animal Care and Use Committees at Children's Hospital, Boston.

Intravitreal Injection and Optic Nerve Crush

For each intravitreal injection, the micropipette was inserted in peripheral retina just behind the ora serrata and was deliberately angled to avoid damage to the lens. The left optic nerve was exposed intraorbitally and crushed with forceps for 5 s approximately 1 mm behind the optic disc, as described previously (Park et al., 2008).

RGC Purification

For retrograde labeling of RGCs, the superior colliculi of adult Sprague-Dawley rats were injected with DiI (2% in DMF). Eyes were removed and retinas were prepared for the cell dissociation procedures 5–7 days after surgery. Dissociated retinal cells were used for FACS sorting to collect DiI-positive RGC cells.

RT-PCR and qRT-PCR

Total RNA was extracted from purified RGCs and was reverse transcribed to cDNA, which was amplified by PCR using specific primers for XBP-1u or XBP-1s. For qRT-PCR, total RNA (50–100 ng) was reverse transcribed and amplified with TagMan predesigned real-time PCR assays. Each sample was run in quadruplicate in each assay. GADPH was used as the endogenous control.

Immunohistochemistry and In Situ Hybridization

Immunostaining and in situ hybridization were performed following standard protocols (Park et al., 2008). Retinal sections were incubated with primary antibodies overnight at 4°C and washed three times for 15 min each with PBS. Secondary antibodies were then applied and incubated for 1 hr at room temperature. Sections were again washed three times for 15 min each with PBS before a coverslip was attached with Fluoromount-G. For RGC counting, whole-mount retinas were immunostained with the TUJ1 antibody, and 6–9 fields were randomly sampled from peripheral region per retina to estimate RGC survival. The people who counted the cells were blinded with the treatment of the samples.

AAV Production

For making AAV2-XBP-1s, we inserted the cDNA of XBP-1s-3HA downstream of the CMV promoter/β-globin intron enhancer in the vector pAAVsc CB6. RBG and viral preparation was made by UMass Gene Therapy Center. The titer determined by silver staining is 1.85×10^{12}.

Induction of Chronic IOP Elevation in Mice

The procedure has been described in detail recently (Chen et al., 2010; Sappington et al., 2010). Briefly, in anesthetized mice, elevation of IOP was induced unilaterally in adult mice by anterior chamber injection of 2 μl fluorescent polystyrene microspheres. The control group received 2 μl saline to the anterior chamber. Mice received a second injection of microbeads at 4 weeks after the first injection. The mice with corneal opacity or signs of inflammation in the anterior chamber (e.g., cloudy anterior chamber) were excluded from further analysis. IOP was measured every other day in both eyes using a TonoLab tonometer.

Statistical Analyses

Data are presented as means ± SEM. We used Student's t test for two group comparisons and one-way analysis of variance and Tukey's multiple comparison test for multiple comparisons.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2011.11.026.

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